BEHAVIORAL AND NEURAL BIOLOGY 25, 380–386 (1979)

Brain Mast Cell Numbers in the Albino Rat: Sources of Variability

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Possible sources of variability in brain mast cell (MC) numbers were investigated experimentally in 36, 21-day-old albino laboratory rats (*Rattus norvegicus*). MC numbers did not differ significantly between brains that had been either: (1) immersed in ethanol–formalin–acetic acid fixative, (2) anesthetized with barbituate and then immersed in fixative, (3) anesthetized and perfused with saline and then fixative, or (4) anesthetized and perfused with saline–heparin and then fixative. Neither variations in perfusion or immersion times (within 35 min) after anesthesia, body weights nor sex significantly contributed to MC numbers. Significant litter differences accounted for 20 and 40% of the variance in MC numbers within the diencephalic leptomeninges and parenchyma, respectively, but did not influence cortical MC numbers. Differences between rats from the same litters accounted for 80 to 60% of the total MC number variance. Estimated total MC numbers within the diencephalic regions of different rats ranged from 3000 to 45,000.

Copious numbers of mast cells (MCs) have been reported in the brains of several dozen mammalian species, including man (Cammermeyer, 1973; Ibrahim, 1974; Dropp, 1976; Kiernan, 1976). The actual numbers of brain MCs within a given species often demonstrate large interindividual variability due to unspecified sources. In the albino laboratory rat (*Rattus norvegicus*), for example, MC numbers within brains may range from almost none to several thousands. Although MC numbers clearly vary as a function of organismic age (Krüger, 1974; Dropp, 1976) and postnatal-preweaning handling procedures (Persinger, 1977), rats from presumably homogeneous origins still display large individual variability. Such variability could be considered serious in experimental designs where single organisms are selected as representative of experimental treatments or particular histories.

The lability of MC numbers as a function of various routine laboratory procedures is not clear. Although MC modifications from environmental

[1] Thanks to Gyslaine Lafrenière for microtome work.

0163-1047/79/030380-07$02.00/0
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or behavioral stimuli have been suggested (Persinger, 1977b), quantitative studies in which care has been taken to specify and maintain likely variables have not been designed experimentally. Frequently, studies involve only one or two subjects within a given treatment or recruit brains with unclear histories. The present study was designed to determine the degree to which brain MC numbers and variability in 21-day-old (preweaned) albino rats were influenced by: (1) four different fixation procedures, (2) between litter differences, (3) within litter differences (differences between individuals from the same litters), (4) time between initiation of fixation procedure and death, and (5) litter disturbance on the kill day. The 21-day-old rat was selected for investigation since: (1) large numbers of MCs (and hence potentially greater ranges of differential alteration to various treatments) occur in this organism and (2) postweaning confounding variables (cage differences, social effects, etc.) are obviated.

Forty 21-day-old Wistar albino rats (*R. norvegicus*) from five different litters were used as subjects. Their parents had been obtained from Bio Breeding Laboratories (Ottawa) and had been bred at 70–74 days of age. Three days before term, the mothers were removed from standard housing and placed in individual 41 × 25 × 18-cm plastic cages containing 0.25 in. corncob (Bedocob) bedding. Within 24 hr after parturition, each litter was culled to eight pups. Pups were not touched except for two mandatory bedding changes on postnatal Days 8 and 16. The following colony conditions existed throughout the study: temperature 22 ± 1°C, relative humidity 45 ± 5%, background noise 70 ± 2 db, and L:D cycle 12:12.

On the kill day, four pups (first kill half) from a given litter were weighed, brought to the laboratory, and allocated to one of four fixation treatments: (1) decapitation followed by whole brain immersion in the fixative (E. F. A., i.e., 90 parts, 80% ethanol; 5 parts, 30% formaldehyde, and 5 parts, glacial acetic acid), (2) barbiturate overdose (ip injection of 3.5 mg sodium phenobarbital) followed by whole brain immersion, (3) barbiturate overdose followed by perfusion with physiological saline (5 ml) and fixative (5 cc), and (4) barbiturate overdose followed by perfusion with heparin–physiological saline (20 mg heparin in 5 ml saline) and fixative (5 ml). Within 2 hr, the remaining four pups (second kill half) of the litter were processed as well; all five litters were treated similarly. Perfusion was completed through the common carotid artery. Since 4 of the 40 rat brains (one from four different litters) did not demonstrate the usual marked blanching after perfusion, they were eliminated from the study. The time between the arrival of the rats at the laboratory (from the colony) and decapitation (treatment 1), barbiturate injection and decapitation (treatment 2), or barbiturate injection and initial perfusion (treatments 3 and 4) ranged from 5 to 35 min and was counterbalanced among treatments so that the average times of the four treatments showed no

statistically significant differences. At the time of decapitation (treatments 1 and 2) or initial perfusion (treatments 3 and 4) atrial activity was still apparent in all subjects. Within 2 min of decapitation, the cerebrums of all rats were removed with minimal mechanical distortion and immersed in fixative for 48 hr.

After dehydration (2 × 2 hr 95% ethanol, 2 hr 100% ethanol, 12 hr 100% ethanol, and 2 hr 100% ethanol), clearing (2 × 1.5 hr chloroform) and paraffin infiltration-bedding (3 × 2 hr) the tissues were sectioned (coronal) at 10 μm. Five sections, equally spaced (i.e., ~650 μm apart) between the posterior commissure and the subfornical organ were selected for each rat and stained for mast cells, as defined by both metachromasia and morphology at 400 ×, with toluidine blue 0 (Humason, 1972). This fixative-staining procedure allowed clear differentiation of MCs (purple to reddish purple) against the pale blue background of the other nuclei (even the occasional darkly stained neuron was easily distinguished). Adult rat thyroid and thymus tissues, stained in the same sequences, also showed the usual number of MCs.

The entire surface area of each section was traversed at 400 × using an Olympus microscope (trinocular Model EC). Total numbers of MCs (recorded on a hand counter) for each 10-μm section were determined in: (1) the leptomeninges, by traversing the perimeter of the diencephalon, (2) the diencephalic parenchyma, (3) the cerebral cortices, and (4) the remaining subcortical structures. To insure accurate counting: (1) boundaries for adjacent circular microscopic fields were followed carefully, (2) care was taken to include MCs in the small areas forming the cusp between adjacent fields, (3) the coaxial stage knobs controlling backward–forward and lateral movements of slide holder were kept tight to prevent drift, and (4) a grid eye piece was used to facilitate MC counting within a field; in addition, two sections, recounted four times each during the cytometric period indicated a replication variability of less than 2%.

The means of MC numbers for each brain were calculated by averaging the values from its five slides. Analyses of variance, analysis of covariance, means, standard deviations, Pearson product moment correlations, and Cochran tests for homogeneity of variance were completed by computer using SPSS programs. Sample analyses were checked manually.

The means and standard deviations for the numbers of MCs/10-μm section [mean total MC numbers within the boundaries measured can be estimated by multiplying the group means by 262, (the number of 10-μm sections cut for each brain)] in: (1) the leptomeninges of the diencephalon, (2) the diencephalic parenchyma, and (3) the cerebral cortices are shown in Table 1 as a function of fixation treatment, litter number, sex, and kill half. Since no MCs were detected in telencephalic subcortical structures, this category is not included. Means and standard deviations for body

weights of pups as a function of these variables are also presented in Table 1.

One-way analysis of variance (ANOVA) demonstrated no statististically significant ($F(3, 32) = <1, P > 0.05$) differences between fixation treatments for MC numbers in the leptomeninges, or in the diencephalic tissue or for body weights. A marginally significant difference was noted between treatments for cortical MC numbers [$F(3, 32) = 3.17, P < 0.05$]. Ad hoc Duncan's multiple range tests set at $P < 0.05$ indicated that the differences were primarily between treatment 1 (no barbiturate) and treatments 2, 3, and 4 (barbiturate anesthesia). However, Cochran's test for homogeneity of variance was significant ($C = 0.690, P < 0.001$). A nonparametric median test (Siegel, 1956) showed that the difference in cortical MC numbers between treatments was not significant [χ^2 (3) = 5.77, $P > 0.05$].

Significant litter differences were noted for MC numbers in the leptomeninges [$F(4, 31) = 5.68, P = 0.001$] and in the diencephalic parenchyma ($F = 2.98, P < 0.05$) but not in the cortices. Significant differences

TABLE 1

Means and Standard Deviations (±) for Mast Cell Number per 10-μm Sections in the Diencephalic Leptomeninges, Diencephalic Parenchyma, and Cerebral Cortices and for Body Weights as a Function of Fixation Treatments, Different Litters, Sexes, and Portion of the Litter Killed First or Second (Kill Halves).

	Diencephalon			
Condition	Leptomeninges	Parenchyma	Cerebral cortices	Body weight (g)
Fixation treatment				
1 (n = 9)	14.6 ± 5.0	66.6 ± 56.1	1.6 ±1.5	50.4 ±3.3
2 (n = 9)	13.5 ± 9.3	49.7 ± 42.5	0.6 ± 0.6	47.0 ± 6.1
3 (n = 9)	11.3 ± 7.0	30.1 ± 15.4	0.3 ± 0.4	50.0 ± 4.2
4 (n = 9)	12.2 ± 4.5	29.2 ± 28.3	1.0 ± 0.8	50.7 ± 6.4
Litter No.				
1 (n = 7)	14.7 ± 6.1	26.8 ± 20.4	0.5 ± 0.3	52.9 ± 2.3
2 (n = 7)	19.6 ± 3.9	68.6 ± 44.0	1.3 ± 1.2	46.4 ± 2.9
3 (n = 7)	13.3 ± 6.9	71.2 ± 48.6	1.0 ± 1.1	46.1 ± 3.0
4 (n = 7)	7.1 ± 4.2	24.4 ± 17.2	0.7 ± 0.5	45.9 ± 4.9
5 (n = 8)	10.2 ± 4.7	30.4 ± 39.3	1.0 ± 1.5	55.5 ± 2.5
Sex				
Male (18)	12.7 ± 6.7	40.8 ± 47.2	0.9 ± 1.0	50.2 ± 6.1
Female (18)	13.1 ± 6.6	46.9 ± 32.9	0.9 ± 1.0	48.8 ± 4.1
Kill half				
1 (n = 16)	12.4 ± 7.4	44.1 ± 43.1	0.9 ± 0.9	49.4 ± 6.4
2 (n = 20)	13.3 ± 5.9	43.4 ± 38.8	0.9 ± 1.1	49.6 ± 4.1

in body weights were evident between litters ($F = 14.36, P < 0.001$). No significant differences were noted between sexes or kill halves for any of the MC measures, kill latencies (variations in perfusion or immersion times after injection of barbiturate) or body weights. No significant differences (all F's ≤ 1) in the experimentally controlled kill latencies existed between fixation treatments, litters, sexes, or kill parts; all mean kill latencies were between 13.9 to 17.0 min with standard deviations between 6.4 and 8.4 min.

Analyses of covariance for individual body weights or kill latencies before ANOVAs demonstrated no significant additional differences in MC measures between fixation treatments (df = 3, 31), litters (df = 4, 30), sexes (df = 1, 33), or kill halves (df = 1, 33). Neither body weights nor kill latencies contributed significantly [$F(1, 35) < 1, P > 0.05$] to the variance of MC numbers. Correlational matrices between the three MC measures, body weights, and kill latencies demonstrated significant correlations between MC numbers: (1) in the leptomeninges and in the diencephalic parenchyma ($r = +0.62, P < 0.001$) and (2) in the latter and in the cortices ($r = +0.68, P < 0.001$). A marginally significant ($P < 0.05$) correlation ($r = +0.28$) existed between MC numbers in the cortices and leptomeninges.

The distributions of MC numbers within brain tissue between the posterior commissure and subfornical organ were not homogeneous. Within the diencephalon, no MCs were ever seen within any hypothalamic nuclei; occasional MCs (not more than two per section) were seen in the pallisades of the median eminence. As shown previously (Dropp, 1976; Persinger, 1977) the longitudinal distribution of MC numbers in thalamic space is bimodal, concentrating primarily in and around the ventral nuclei and the anteroventral nuclei. Occasional clusters were found in the reticular nuclei and parafascicular nuclei. Analysis of cortical MCs indicated that 98% of the 161 counted in 180 sections (5 × 36 rats) appeared in the parietal cortices within layers III and IV; the remaining MCs occurred in the pyriform cortices. As mentioned by Dropp (1974) the majority of MCs occurred around blood vessels. MC clusters invariably occurred along blood vessels with major axes oriented along the section's plane. The only displays of "degranulation" were the occasional metachromatic granules immediately proximal to the cell membrane. Metachromatic artifacts (varying-sized metachromatic granules or globs within blood vessels) were noted in two of the nine saline–heparin perfused brains only. Estimates of total MC numbers within diencephalic areas demonstrated mean litter ranges between 8000 and 22,000 MCs and minimum–maximum values for all 36 rats between 3000 and 45,000 MCs. The group means are approximately two to five times greater than the values that were extrapolated from Dropp's (1976) data.

The results clearly indicate that the fixation treatments employed in this

study did not produce large differences in brain MC numbers. When the heterogeneity of group variances was considered (for the cortical MC numbers) even marginally statistically significant differences did not exist between brains that had been either: (1) removed following decapitation and immersed in fixative, (2) removed following barbiturate anesthesia and immersed in fixative, (3) perfused with saline and then fixative following the anesthesia, or (4) perfused with saline–heparin and then fixative following the anesthesia.

The stability of MC numbers is also indicated by the absence of significant alterations following routine laboratory or experimental manipulations. MC numbers were not significantly altered by differential decapitation or perfusion times that ranged from 5 to 35 min following introduction to the laboratory or anesthesia. Since there were no significant differences in any of the MC measurements between the pups killed within 35 min after initial disturbance of the nest (first kill half) and those killed about 2 hr later (second kill half), one can conclude that such behavioral stimuli do not alter MC numbers.

Significant litter differences were apparent for MC numbers within diencephalic regions but not within the cortices. ω^2 estimates for proportion of variances indicated that litter differences accounted for 20 and 40% of MC variance in the thalamic nuclei and leptomeninges, respectively. Sex differences did not contribute in any significant manner to MC number variation.

By far the greatest source of variance in MC numbers occurred *within* litters. Variance estimates indicated that 80% of the parenchymal diencephalic MCs and 60% of the leptomeningeal MCs were due to pup differences within the same litter. Since variance differences between the five litters were not statistically significant, such large within-litter variability appears reliable. Considering the carefully controlled laboratory histories of the litters, the source of variance should be related to as yet unspecified congenital/maternal factors.

The results of this study may help clarify two aspects of mast cell-related research. First, the large variability of MC numbers within animals from the same litters (and without complex and different postweaning histories) may help explain the discrepancies between past accounts of MCs in the rat brain. Studies in which one or two rats are selected as representatives of various experimental manipulations should be viewed with caution. Second, if young rat brain histamine is primarily contained within mast cells as Schwartz (1975) suggests, then the variable concentration of this biogenic amine in single rat brains (Green, 1970) may reflect MC number variability rather than, or as well as, methodological differences.

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